Bradykinin Stimulation of Nitric Oxide Production is not Sufficient for Gamma-Globin Induction

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SUMMARY

Introduction Hydroxycarbamide, used in therapy of hemoglobinopathies, enhances nitric oxide (NO) production both in primary human umbilical vein endothelial cells (HUVECs) and human bone marrow endothelial cell line (TrHBMEC). Moreover, NO increases γ-globin and fetal hemoglobin levels in human erythroid progenitors.

Objective In order to find out whether simple physiologic stimulation of NO production by components of hematopoietic microenvironment can increase γ-globin gene expression, the effects of NO-inducer bradykinin were examined in endothelial cells.

Methods The study was performed in co-cultures of human erythroid progenitors, TrHBMEC and HUVECs by ozone-based chemiluminescent determination of NO and real-time quantitative RT-PCR.

Results In accordance with previous reports, the endogenous factor bradykinin increased endothelial cell production of NO in a dose- and time-dependent manner (0.1-0.6 μM up to 30 minutes). This induction of NO in HUVECs and TrHBMEC by bradykinin was blocked by competitive inhibitors of NO synthase (NOS), demonstrating NOS-dependence. It has been shown that bradykinin significantly reduced endothelial NOS (eNOS) mRNA level and eNOS/ß-actin ratio in HUVEC (by twofold). In addition, bradykinin failed to increase γ-globin mRNA expression in erythroid progenitors only, as well as in co-culture studies of erythroid progenitors with TrHBMEC and HUVEC after 24 hours of treatment. Furthermore, bradykinin did not induce γ/β globin ratio in erythroid progenitors in co-cultures with HUVEC.

Conclusion Bradykinin mediated eNOS activation leads to short time and low NO production in endothelial cells, insufficient to induce γ-globin gene expression. These results emphasized the significance of elevated and extended NO production in augmentation of γ-globin gene expression.

Keywords: bradykinin; endothelial cells, erythroid progenitors, nitric oxide, gamma-globin

INTRODUCTION

Nitric oxide (NO) is an important intracellular and intercellular signaling molecule with a significant role in maintaining normal blood pressure in vivo. The production of NO in the body, from the non-aromatic amino acid L-arginine and molecular oxygen, is catalyzed by a family of enzymes called NO synthases (NOSs). One of them is endothelial NOS (eNOS), expressed in endothelial cells, cardiac myocytes and blood platelets. eNOS activity is controlled at the transcriptional and post-transcriptional levels as well as at the post-translational level [1]. One of the agonists that contribute to activation of eNOS in endothelial cells is bradykinin, a potent inducer of eNOS activity [2]. Bradykinin is an endogenous non-peptide derived from proteolysis of higher molecular weight precursor proteins synthesized in the liver and hypothalamus. Bradykinin acts as a local hormone whose pharmacological actions include hypertension, increase in vascular permeability and blood coagulation [3]. In addition, it is another key determinant of vascular function since its B2 receptors in endothelial cells modulate vasodilation, vascular permeability, mitogenesis and adhesion molecule expression [4].

Hydroxycarbamide has demonstrated efficacy in adults with sickle cell disease. Hydroxycarbamide treatment is associated with fewer vaso-occlusive pain episodes and hospitalizations, and lower total costs of care. The incidence of painful crises has been reduced significantly, as well as the rates of the acute chest syndrome and blood transfusion [5]. Long-term follow-up showed 40% reduction in mortality among those who received hydroxycarbamide [6]. In sickle cell disease, the protective effect of fetal hemoglobin (HbF) is mediated primarily by decreasing intravascular sickling, resulting in the increased NO bioavailability [7]. Moreover, adult hemoglobin (HbA) is expressed in human arterial endothelial cells and augmented at the myoendothelial junction, where regulates the NO effects on vascular reactivity. The endothelial HbA hem iron in the ferric Fe^{3+} status allows NO signaling, and this signaling is not active when HbA is reduced to the ferrous Fe^{2+} condition [8]. According to our previous results, HbF inducer hydroxycarbamide increased NO production in both pri-

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mary human umbilical endothelial cells (HUVECs) and human endothelial cell line (TrHBMEC) through eNOS phosphorylation [9]. Moreover, during human erythroid differentiation *in vitro*, eNOS mRNA and protein levels were initially high but then declined steadily, as did the production of NO derivatives [10]. In addition, NO increased γ-globin levels, the γ/ß-globin ratio and HbF levels in human erythroid cells [11].

OBJECTIVE

The erythroid progenitors in co-cultures with hematopoietic stromal cells, either macrophages or endothelial cells stimulated by NO-inducer hydroxycarbamide, demonstrated increased γ-globin gene expression in comparison to the erythroid cells only [12]. Therefore, NO mediated stimulation may have resources in the microenvironment supported by delivered endogenous factors, besides exogenous treatment. To explore possibility whether physiologic stimulation of constitutive NO production by hematopoietic microenvironment can influence γ-globin gene expression, the effects of bradykinin were investigated in co-cultures of human endothelial and erythroid progenitor cells.

METHODS

Endothelial cell cultures

HUVECs (BioWhittaker Inc., Walkersville, MD, USA) were cultured in endothelial cell growth medium (EGM, BioWhittaker Inc.) at 37°C in a humidified environment containing 5% CO_2. The cells from the 3rd and 4th passages were used in the present experiments. TrHBMECs were continuous bone marrow endothelial cell line from an adult female donor cells immortalized with the T antigen of simian virus 40 [9]. TrHBMECs were cultured in culture flasks and dishes on 0.2% gelatin (Sigma-Aldrich, St Louis, MO, USA) at 37°C in Dulbecco's modified essential medium-low glucose (DMEM, Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Gemini BioProducts, Woodland, CA), 100U/ml penicillin, 100μg/ml streptomycin, 1 μg/ml folic acid (Sigma-Aldrich), and 3mM glutamine (Biofluids, Gaithersburg, MD, USA). Experiments were done between passages 19 and 23. The EGM medium for HUVEC and DMEM medium for TrHBMEC already contained 0.36 and 0.48 mM l-arginine, respectively. HUVECs and TrHBMECs were treated with bradykinin (Enzo Life Sciences Inc., Farmingdale, NY, USA) at concentrations indicated in Graphs, and at different time points, confluence was determined by inverted microscope. Additives were left throughout the periods of the culture. The viable cell counts were performed with the use of the Trypan-blue exclusion technique (BioWhittaker Inc.).

Co-culture studies

A two-phase liquid culture protocol for erythroid cells was performed as previously described [11]. Briefly, peripheral blood-derived CD34$^+$ cells were harvested and purified by negative selection using the StemSep Cell Separation method (Stem Cell Technologies Inc, Vancouver, Canada). The CD34$^+$ cells were resuspended in the phase II medium which contained 30% FBS, 2 mmol/l glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% deionized BSA, 10 μmol/l β-mercaptoethanol, 1 μmol/l dexamethasone, 33 μg/ml holo-transferrin, 10 ng/ml stem cell factor (SCF), 1 ng/ml IL-3, 1 ng/ml GM-CSF (Sigma-Aldrich), and 1 U/ml human recombinant erythropoietin (Amgen Inc., Thousand Oaks, CA, USA) to stimulate erythroid differentiation. The adherent endothelial cell cultures were prepared in 6-well plates. Co-cultures of erythroid progenitor cells with TrHBMEC and HUVEC were treated with bradykinin (Enzo Life Sciences Inc.). The inserts containing erythroid progenitors were placed in the wells containing endothelial cells, but separated from them by 1μm-pore semipermeable membrane (Biocoat BD Biosciences Discovery Labware, Bedford, MA, USA), while sharing the same phase II medium. Erythroid progenitor cells were placed in co-cultures on day 4, after initiation of phase II culture, and incubated for 24 hours in phase II medium at 37°C in the humidified atmosphere with 5% CO_2 during treatment by bradykinin. After incubation with bradykinin, the supernatants were transferred to black microplates and the fluorescence of extracellular NO/DAF-2 interaction was measured with a fluorescence microplate reader calibrated for excitation at 470 nm and emission at 550 nm. The intracellular NO levels were measured by the cell permeable probe 4,5-diaminofluorescein diacetate (DAF-2DA, Enzo Life Sciences Inc.) using a microplate reader and Epics Elite flow cytometer (Coulter, Hialeah, FL, USA).

Ozone-based chemiluminescent determination of nitrite

For nitrite measurements, confluent HUVECs in 6-well plates were washed once with HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (BioWhittaker Inc.) and endothelial basal medium (EBM) was added. After 3 hours, the medium was replaced with a new EBM and cells were treated with bradykinin. Confluent TrHBMECs in 6-well plates were washed once, and the medium was replaced with DMEM supplemented with 100U/ml penicillin, 100μg/ml streptomycin, 1μg/ml folic acid and 3mM glutamine. The cells were left overnight at 37°C (glutamine was removed for eNOS inhibitor experiments). The following day, the medium was replaced with DMEM supplemented with 100U/ml penicillin, 100μg/ml streptomycin and 1μg/ml folic acid. For the eNOS inhibitors, TrHBMECs and HUVECs were pretreated for 30 minutes with N^G-Nitro-L-arginine-methyl ester (L-NAME) and N^G-Nitro-l-arginine (Enzo Life Sciences Inc.), respectively, and then exposed to bradykinin. The supernatant of

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treated cells was immediately frozen on dry ice and stored at -80°C. Frozen samples were thawed and then the level of nitrite was determined with the Sievers Model 280 NO analyzer (Sievers, Boulder, CO, USA). NO was measured by nitrite reduction in acidified KI containing 7 ml of glacial acetic acid, 2ml of distilled water, 50 mg of KI and a crystal of iodine that was added to yield a concentration of 6-20 mmol/l (Sigma-Aldrich). Helium was bubbled through the reaction mixture.

RNA extraction and real-time quantitative reverse transcriptase PCR

For isolation of total RNA from erythroid cells, the RNeasy procedure was used (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The concentration and integrity of total RNA were assessed using an 8453 UV/Visible Spectrophotometer (Hewlett-Packard GmbH, Waldbronn, Germany) and Agilent 2100 Bioanalyzer Software (Agilent Technologies, Waldbronn, Germany). One microgram of total RNA was reverse transcribed with SuperScript II RNase H^- Reverse Transcriptase kit (Life Technologies). The specific primers of human γ- and β-globin, eNOS genes as well as TAQMAN probes were designed using Primer Express software and prepared on the ABI 394 synthesizer as previously described [11, 13]. Platinum Quantitative PCR SuperMix-UDG (Life Technologies) was used, containing final concentration of 200 μM 2'-deoxynucleoside 5'-triphosphates (dNTPs), 0.5 μM Rox reference dye (Life Technologies), 0.2 μM each of TAQMAN probe, sense and antisense primers. Expression levels were determined using the associated SDS software (ABI Prism, Applied Biosystems) and Microsoft Excel (Redmond, WA, USA).

Statistical analysis

The one-way analysis of variance (ANOVA) and Dunnett's post tests were applied using Prism 4 software (GraphPad Software Inc., San Diego, CA, USA). For comparison of bradykinin effects on erythroid progenitor cells, with or without TrHBMEC and HUVEC, the paired t-test was used with two-tail P values. The paired t-test was also used for comparison of bradykinin induction of NO levels with or without L-NAME and N^G-Nitro-L-arginine.

RESULTS

Bradykinin increases NO production in endothelial cells

It has been previously demonstrated that bradykinin stimulated eNOS phosphorylation in HUVEC [9]. These studies were expanded to study the effect of known NO agonist bradykinin in endothelial cells, with a high level of the eNOS protein [2]. TrHBMEC were treated with different concentrations of bradykinin (0-20 μM) and NO production was measured at different time points, during the incubation period of 60 minutes. Bradykinin induced NO production in a dose- and time-dependent manner in TrHBMEC (Graph 1A). A more prominent elevation of NO levels, by bradykinin, was observed during first 15 minutes of treatment in cultures with TrHBMEC. After that, there was a sudden decrease in NO levels within 60 minutes of incubation. Similar and consistent dose-dependent increase in NO production, with an early peak after 15 minutes, was also obtained during the treatment of HUVEC with 20 μM bradykinin and L-arginine (Graph 1B). Reduced 10 μM dose of bradykinin also increased NO production, but with postponed peak after 30 minutes in HUVEC (Graph 1B). NO measurement was prolonged up to 6 hours of incubation and elevation of NO levels in HUVEC during bradykinin treatment was not determined (not shown). Therefore, bradykinin stimulated NO production was confirmed in presented endothelial cell culture model.

Inhibition of eNOS prevented NO induction by bradykinin in endothelial cells

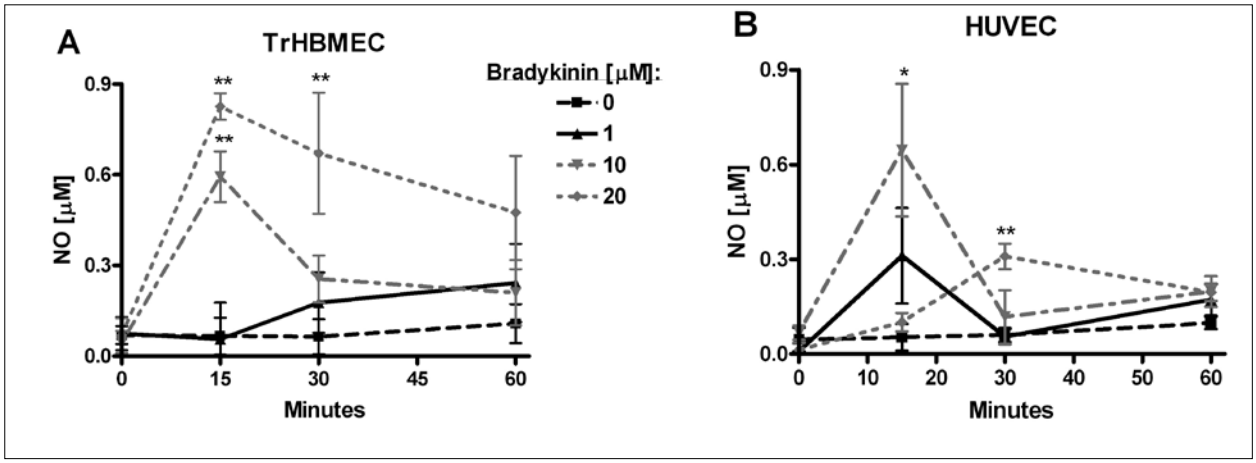
It has been already demonstrated that bradykinin stimulation of eNOS phosphorylation/activation was prevented by the NOS inhibitor L-NAME in HUVEC [14]. To check NOS dependence during bradykinin stimulation of NO production, TrHBMEC and HUVEC cells were pretreated with the NOS inhibitors. Increased NO production in endothelial cells, during incubation with bradykinin, was blocked by competitive inhibitors of NOS such as L-NAME and N^G-Nitro-L-arginine. TrHBMEC, preincubated with L-NAME, demonstrated remarkably inhibited induction of NO by bradykinin (Graph 2A) during first 30 minutes. Similar results were obtained with HUVEC treated with N^G-Nitro-L-arginine (Graph 2B). Thus, the NOS dependence was confirmed in bradykinin stimulation of NO production in the existing endothelial cell culture model.

Bradykinin decreases eNOS mRNA levels in endothelial cells

In order to test whether increase in eNOS mRNA level was responsible for the increase in NOS activity, eNOS mRNA levels were measured after 24 hours of HUVEC incubation with bradykinin. According to our experiments, bradykinin inhibited eNOS mRNA levels without a dose dependent effect in HUVEC (Graph 3A). In addition, bradykinin also reduced the eNOS/β-actin ratio in HUVEC after 24 hours of treatment (Graph 3B). Therefore, the bradykinin induction of eNOS activity was not controlled at transcriptional level.

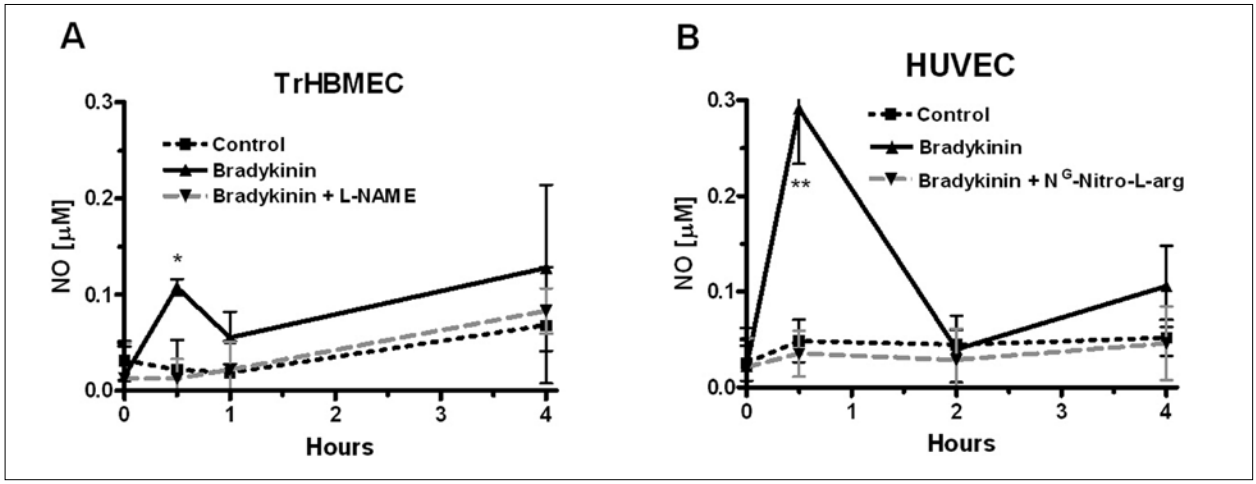
Induction of NO production in TrHBMEC and erythroid progenitors by bradykinin

It has been shown that bradykinin stimulated NO production in erythroid progenitors [8]. In the following experi-



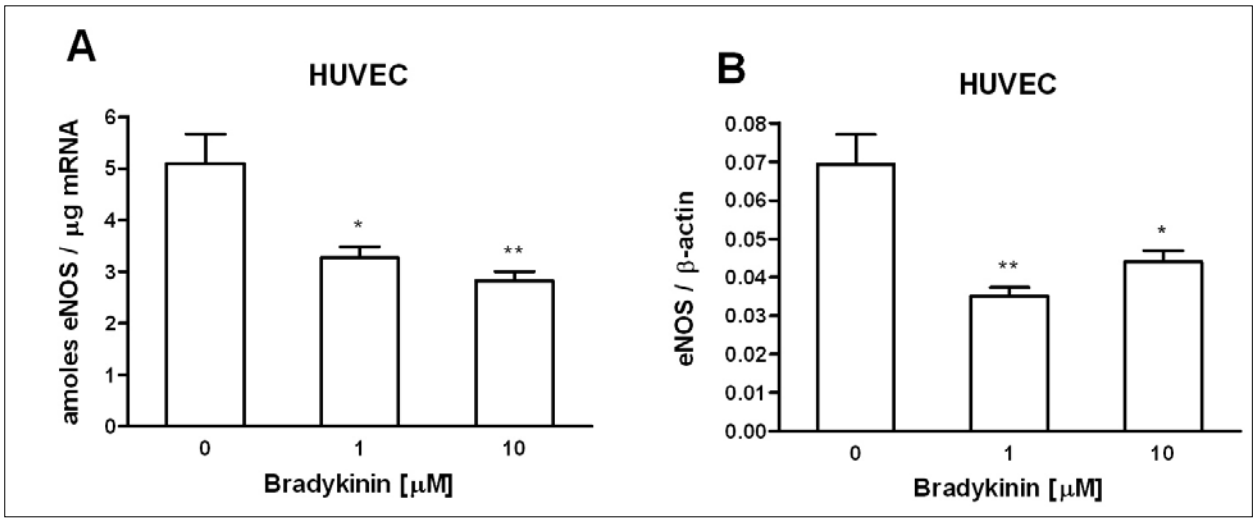
Graph 1. Bradykinin stimulated NO production in endothelial cells: A) bradykinin treatment of TrHBMEC with addition of 1mM l-arginine; B) bradykinin treatment of HUVEC with addition of 1mM l-arginine during 60 minutes of incubation

Values represent means ± SEM (n=3).
* p<0.05 and ** p<0.01 versus untreated cells



Graph 2. Bradykinin NOS-dependently increased NO levels in endothelial cells: A) Bradykinin (10 μM) stimulation of NO production was prevented by NOS inhibitor L-NAME (0.5 mM) in TrHBMEC during the first 30 minutes of treatment; B) Bradykinin (10 μM) stimulation of NO production was prevented by NOS inhibitor NG-Nitro-l-arginine (0.5 mM) in HUVEC during the first 30 minutes of treatment.

Values represent means ± SEM (n=3).
* p<0.05 and ** p<0.01 versus untreated cells



Graph 3. Bradykinin decreased eNOS transcript in endothelial cells: A) bradykinin decreased eNOS mRNA levels in HUVEC after 24 hours of treatment; B) bradykinin decreased eNOS / β-actin ratio in HUVEC after 24 hours of treatment

Values represent means ± SEM (n=3).
* p<0.05 and ** p<0.01 versus untreated cells

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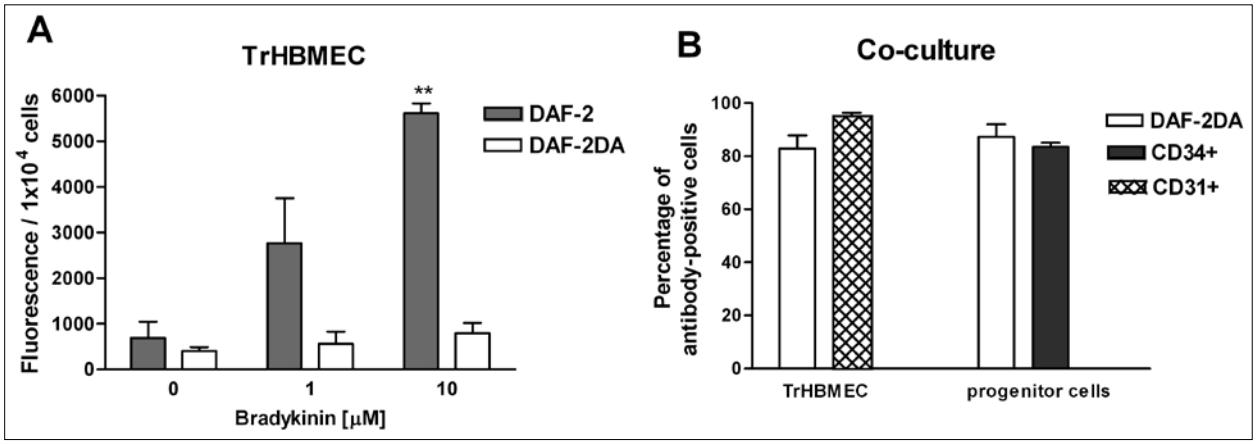
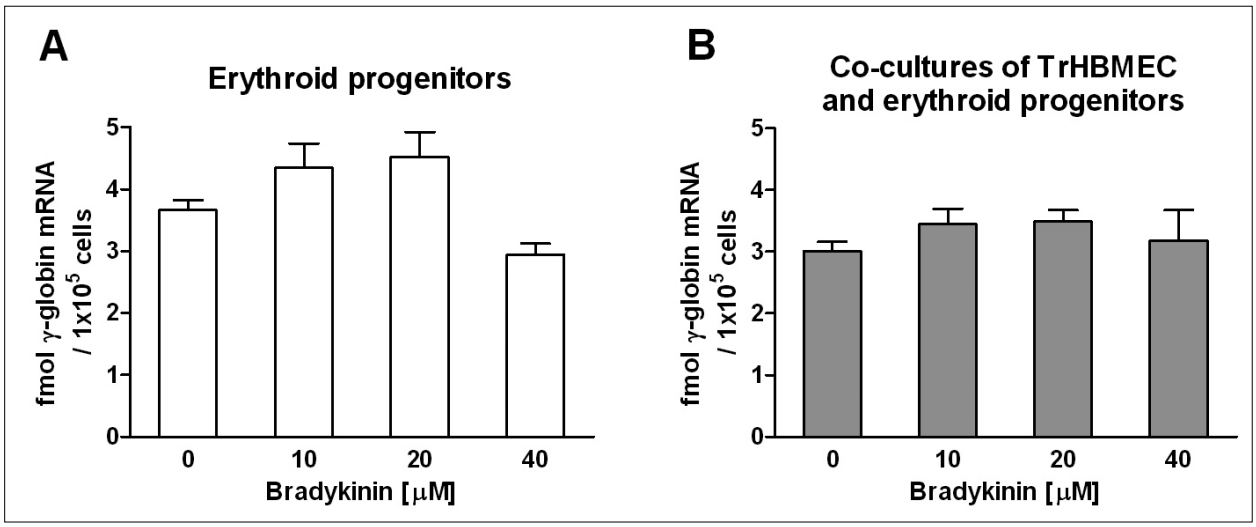


Graph 4. Stimulation of NO production in TrHBMEC and erythroid progenitors by bradykinin: A) Detection of extracellular and intracellular NO levels in TrHBMEC with fluorescent indicators DAF-2 and DAF-2DA (per 10 μM, $1x10^4$ cells) during bradykinin treatment, respectively; B) Flow cytometry of hematopoietic progenitor cells ($2x10^5$, on day 6) by CD34+ antibody and TrHBMEC ($1x10^5$) by CD31+ antibody, plus DAF-2DA (10 μM), in co-culture system treated with 1 mM L-arginine and 10 μM bradykinin.

Values represent means ± SEM (n=3).
** p<0.01 compared to untreated cells



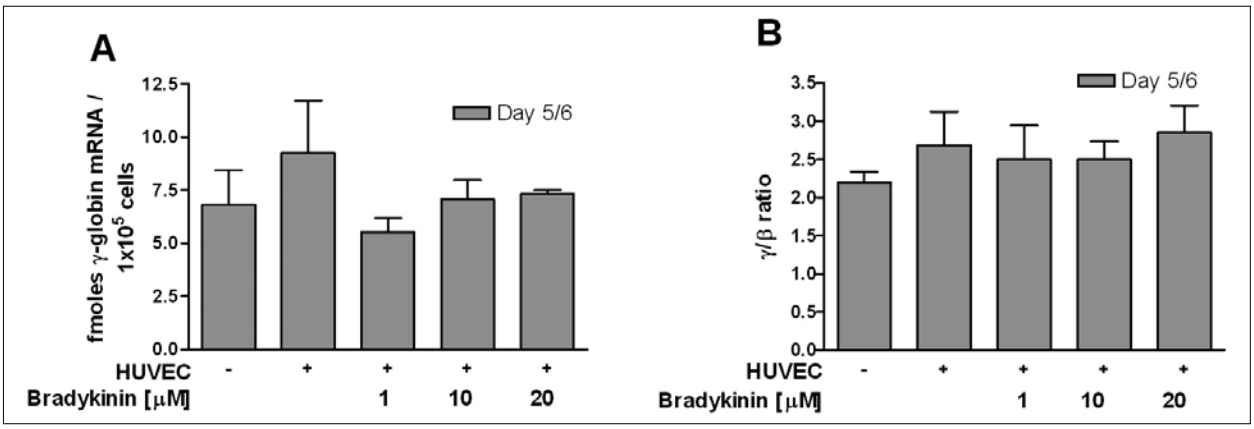
Graph 5. Bradykinin induction of γ-globin gene expression in erythroid progenitors: A) Gamma-globin gene expression in erythroid progenitors during treatment with bradykinin on day 5/6 of culture; B) Gamma-globin gene expression in co-cultures of TrHBMEC and erythroid progenitors during treatment with bradykinin on day 5/6 of culture

Values represent means ± SEM (n=3).

ments, the induction of extracellular and intracellular production of NO by bradykinin was observed in TrHBMEC and erythroid progenitors during co-culture studies. First, a dose-dependent increase in extracellular NO levels was demonstrated in TrHBMEC during stimulation by bradykinin as measured by fluorescent probe DAF-2 (Graph 4A). Intracellular NO levels, determined by the cell-permeable DAF-2DA probe, were low and did not change significantly during bradykinin stimulation (Graph 4A). To examine intracellular NO presence in both erythroid progenitors and TrHBMEC during co-culture studies and bradykinin stimulation, flow cytometry was used to label simultaneously the erythroid progenitors by $CD34^+$ and TrHBMEC by $CD31^+$ antibody as well as with the fluorescent probe DAF-2DA (Graph 4B). The labeled TrHBMEC were highly enriched with intracellular NO (about 80%) and the same applied for erythroid progenitors during stimulation by bradykinin in co-cultures (Graph 4B).

Gamma-globin gene expression of stimulated erythroid progenitors in co-culture with endothelial cells

As already demonstrated, hydroxycarbamide significantly increased γ-globin mRNA expression in erythroid progenitors of co-culture experiments with TrHBMEC and that increase was higher in comparison with hydroxycarbamide effect on erythroid progenitors only [12]. To examine the general ability of endothelial cell-derived NO to affect γ-globin gene expression, the erythroid progenitors were stimulated by bradykinin. Low- and short-term induced NO production (up to 0.08 μM within first 15-30 minutes) by bradykinin (Graphs 1 and 2) failed to increase γ-globin mRNA expression in erythroid progenitor cells only (Graph 5A), as well as in co-culture of erythroid progenitors with TrHBMEC (Graph 5B) or HUVEC (Graph 6A) after 24 hours of treatment. It has been also shown that



Graph 6. Bradykinin induction of γ-globin gene expression in co-cultures of erythroid progenitors and endothelial cells: A) Gamma-globin gene expression in co-cultures of HUVEC and erythroid progenitors during treatment with bradykinin on day 5/6 of culture; B) Gamma/beta-globin ratio in co-cultures of HUVEC and erythroid progenitors during 24 hours of treatment with bradykinin on day 6 of culture

Values represent means ± SEM (n=3).

bradykinin failed to increase γ/ß ratio in erythroid progenitor cells co-cultured with HUVEC after 24 hours (Graph 6B). Therefore, the endothelial cell-derived NO, induced by bradykinin, was not sufficient to increase γ-globin gene expression in erythroid progenitor cells.

DISCUSSION

It has been demonstrated that bradykinin dose- and time-dependently increased NO production in TrHBMEC and HUVEC. Generally, the effects of bradykinin were similar in both types of endothelial cells. Moreover, NO production in endothelial cells was prevented by NOS inhibitors. Bradykinin did not appear to increase eNOS protein levels by increasing eNOS transcription. After 24 hour-treatment of HUVEC, bradykinin inhibited eNOS mRNA levels. Concomitant increase of NO levels was observed in TrHBMEC co-cultured with erythroid progenitors. Bradykinin failed to increase γ-globin expression in erythroid progenitor cells only as well as in co-cultures of erythroid and TrHBMEC or HUVEC cells. In addition, bradykinin did not increase γ/β ratio in co-culture of erythroid progenitors and HUVEC. According to these results, the NO production induced by physiological stimulator bradykinin was not sufficient for enhancement of γ-globin gene expression.

It has been reported that cGMP-dependent signaling may be important for erythroid HbF induction. High gene expression of cGMP-specific phosphodiesterase 9A (PDE9A) was found in $CD34^+$-derived erythroid cells and K562 erythroleukemic cells, indicating a high hematopoietic cell expression [15]. Inhibition of PDE9A enzyme significantly increased production of the γ-globin gene in K562 cells [15]. In addition, inhibition of PDE9 amplified the cGMP-elevating effects of hydroxycarbamide [16]. According to early studies, γ-globin mRNA levels were unaffected by NO production of K562 erythroleukemic cells retrovirally transduced with inducible NOS (iNOS), while γ-globin protein expression was reduced in the NO-producing cells in relation to the amount of produced NO [17]. NO did not directly inhibit hemoglobin synthesis, but likely acted gradually in heme synthesis [18]. Recently, it has been shown that increased expression of HbF was accompanied by increased levels of NO metabolites (NOx) and bioavailability [7]. Sickle cell anemia patients with high levels of HbF showed the concomitant elevation of serum levels of NOx [19]. The direct stimulation of cellular NO and cGMP signaling in erythroid progenitors acted as possible mechanism of γ-globin gene activation [20]. It has been reported that NOS substrate arginine dose-dependently increased BFU-E colonies without affecting HbF synthesis. However, the combination of arginine and hydroxycarbamide increased HbF synthesis when compared with hydroxycarbamide treatment only. This synergistic effect on HbF synthesis in human erythroid progenitors was NO dependent [21]. According to our results, the NO inducer bradykinin did not stimulate γ-globin gene expression in erythroid progenitors. Therefore, both NOS substrate arginine and physiologic stimulator bradykinin were not sufficient for enhancement of γ-globin gene expression in two independent studies.

The bradykinin receptor type 2 (B2R) was expressed constitutively in endothelial cells, while B1R was upregulated by inflammatory mediators. B2R-mediated eNOS activation led to lower and transient (about 5 min.) output of NO in endothelial cells whereas in cytokine-treated endothelial cells, B1R activation led to very high and prolonged (about 90 min.) NO production that was mediated by post-translational activation of iNOS [22]. According to our previous reports, bradykinin-induced NO production lasted 15-30 min. in endothelial cells, while butyric acid and hydroxycarbamide stimulated a protracted NO production, enduring couple of hours to couple of days [9, 13]. B2R coupling to Gαq/11 or Gαi/o resulted in downstream activation of PLC, leading to the formation of diacylglycerol and inositol 1,4,5-trisphosphate (IP3), which increased cytoplasmic Ca^{2+} concentrations involved in the generation of NO. It has been already proposed that γ-globin stimulation was regulated via G proteins of the Gαs (stimulation of cAMP/PKA) and Gαq (stimulation of intracellular Ca^{2+}) families [23]. Therefore, bradykinin

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just partially activated Gαq and, furthermore stimulated a reverse Gαi (inhibited cAMP/PKA pathway). Bradykinin induced biphasic rise in intracellular Ca^{2+}, an initial transient peak generated from the intracellular stores, followed by the sustained plateau phase caused by extracellular Ca^{2+} entry through calcium channels in the plasma membrane [24]. In our previous report, hydroxycarbamide increased intracellular Ca^{2+} after 2-6 minutes and generally had a protracted effect in endothelial cells [9]. Stimulation of eNOS phosphorylation at Ser1177 and dephosphorylation at Thr495 also resulted from B2R stimulation [25]. A transient, about 5 min., eNOS phosphorylation at Ser1177 by bradykinin, as well as by hydroxycarbamide, has been already observed [9]. This comparative review demonstrated that bradykinin just partially fulfilled activities of standard γ-globin inducers, such as hydroxycarbamide.

Hydroxycarbamide increased HbF levels through the NO-dependent activation of soluble guanylyl cyclase in erythroid cells, while NO increased γ-globin levels, γ/β-globin ratio and HbF levels in human erythroid cells [11]. Co-culture of human bone marrow endothelial cells and erythroid progenitor cells also induced γ-globin mRNA expression in the presence of hydroxycarbamide. The persistent and modest NO production (between 0.6 and 1.3 μM), by stimulated hematopoietic stromal cells, preferably increased γ-globin gene expression. The erythroid progenitor cells co-cultured either with macrophages or endothelial cells stimulated by iNOS inducers and hydroxycarbamide, respectively, led to elevated levels of γ-globin gene expression as compared to the erythroid cells only [12]. This supplemented NO production, in the hematopoietic microenvironment, has the potential to increase HbF synthesis in erythroid progenitors with low hemoglobin quantity and apparently low NO scavenging activity.

CONCLUSION

According to presented results, bradykinin did not change significantly γ-globin gene expression in solitary erythroid progenitors and in co-cultures with endothelial cells. These findings demonstrated that determined physiologic stimulation of the constitutive NO production was not sufficient for γ-globin gene induction. Further studies should explore the activity of individual G proteins related to G protein coupled receptors during stimulation of γ-globin expression, because eNOS was regulated by reversible and inhibitory interactions with G-protein-coupled receptors in endothelial cells [26]. Using this approach, the upward part of NO/cAMP and NO/cGMP signaling pathways will be described with the accent to explore their activities between membrane receptors and nucleus. This report will support potential studies of hematopoietic microenvironment to explore a therapy for hemoglobinopathies currently treated with hydroxycarbamide.

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Стварање азот-моноксида изазвано брадикинином није довољно за индукцију гама-глобина

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КРАТАК САДРЖАЈ

Увод Хидроксикарбамид, који се користи у лечењу хемоглобинопатија, подстиче стварање азот-моноксида (*NO*) како у примарним људским ендотелним ћелијама пупчане вене (*HUVEC*), тако и у измењеној ендотелној ћелијској линији пореклом из коштане сржи (*TrHBMEC*). Штавише, *NO* повећава стварање γ-глобина и феталног хемоглобина у људским прогениторима еритропоезе.

Циљ рада Да бисмо установили да ли једноставна физиолошка стимулација стварања *NO* од компоненти микросредине хематопоезе може повећати експресију γ-глобинског гена, испитивали смо ефекте брадикинина, већ познатог стимулатора стварања *NO*.

Методе рада Студија је изведена у заједничким културама људских прогенитора еритропоезе са *TrHBMEC* или *HUVEC* и испитивана хемилуминисцентним мерењем *NO* посредством озона, као и применом квантитативног *RT-PCR* на генском нивоу.

Резултати У складу с претходним извештајима, показали смо да ендогени фактор брадикинин повећава стварање *NO* у ендотелним ћелијама на дозно и временски зависан начин (0,1–0,6 *μM* до 30 минута). Ово стварање *NO* у *HUVEC* и *TrHBMEC* изазвано брадикинином блокирано је од стране конкурентских инхибитора *NO*-синтазе (*NOS*), показујући *NOS*-зависност. Утврдили смо да брадикинин значајно смањује стварање иРНК ендотелне форме *NOS* (*eNOS*), као и однос *eNOS* и β-актина у *HUVEC* (двоструко мање). Поред тога, брадикинин не повећава експресију иРНК γ-глобинског гена ни у засебним прогениторима еритропоезе, нити у заједничким културама прогенитора еритропоезе са *TrHBMEC* или *HUVEC* после 24 сата третмана. Брадикинин не мења ни однос γ и β глобина у заједничким културама прогенитора еритропоезе са *HUVEC*.

Закључак Активација *eNOS* изазвана брадикинином доводи до кратког и малог повећања *NO* у ендотелним ћелијама, што је недовољно да подстакне експресију гена за γ-глобин. Ови резултати наглашавају важност повећаног и продуженог стварања *NO* ради стимулације експресије γ-глобина.

Кључне речи: брадикинин; ендотелне ћелије; прогенитори еритропоезе; азот-моноксид; гама-глобин

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